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(54) Title: ELECTRIC FIELD MEDIATED DNA TRANSFORMATION OF PLANT CELLS AND ORGANELLES

(57) Abstract

A method for introducing DNA into plant cells and organelles by means of high-voltage electroporation.

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# ELECTRIC FIELD MEDIATED DNA TRANSFORMATION OF PLANT CELLS AND ORGANELLES

## BACKGROUND OF THE INVENTION

Improvement of heritable characteristics of plants in general and crop plants in specific has classically been effected by genetic crosses. That is, the pollen of one plant has been used to fertilize the eggs of another plant, thereby allowing for the possibility of selecting a plant which displays the good qualities of each of the plants. Such classical genetic manipulation depends upon the ability of the two plants to interbreed.

If a beneficial characteristic exists in a plant, and it is desirable for a second plant to acquire that characteristic, the two plants must be able to hybridize (i.e., interbreed). However, even then the progeny may not display the desirable characteristics of both parents or, if they do, other qualities may be effected or impaired. It would be advantageous, therefore, if there were a means of introducing a specific characteristic into a plant so that one could effect plant improvement in a planned and predictable way.

Currently, efforts are being made to introduce a specific characteristic into a higher plant by co-cultivation of plant cells with bacteria of the genus Agrobacterium. Such bacteria transfer plasmids to the plant cells, a specific portion of which (the T-DNA) gets incorporated into the plant genome. By engineering the gene (DNA) for a desired characteristic into the T-DNA portion of an Agrobacterium plasmid, one can subsequently introduce that gene into a plant in a stable way. However, only certain dicotyledonous plants are susceptible to infection by Agrobacterium. There is a need for a general method for introducing DNA into plant cells of a variety of types.

## SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for introducing DNA into plant cells and organelles.

It is a further object of the present invention to provide a method for stably introducing genes to plant cells and organelles.

It is a further object of the present invention to provide a method for generating whole plants which have stably incorporated, introduced DNA.

It is yet another object to provide a method of introducing DNA into plants which are able to transmit the introduced DNA to succeeding generations.

In accordance with the above objects, one aspect of the present invention provides a method of introducing DNA into plant cells or organelles comprising mixing the plant cells or organelles and the DNA in a suitable medium, and treating the plant cells and organelles and DNA mixture by subjecting it to a pulsed electric field of at least about 0.01 kV/cm.

In another embodiment, the electrically treated mixture of plant organelles and DNA is reintroduced into plant cells.

In another embodiment, the electrically treated mixture of plant cells and DNA is incubated in the presence of plant harmones to provide a transformed plant cell.

The present invention provides a method of introducing DNA into plant cells which can be regenerated into plantlets and which can transmit the introduced DNA to succeeding generations. The present invention can be employed to introduce DNA into either monocotyledonous or dicotyledonous plants or their organelles.

## DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, high-voltage electrical pulses are applied to plant cells or organelles in the presence of exogenous DNA. Such electrical treatment induces a change in the permeability of the plant cell or organelle membrane to DNA, resulting in an increase in the amount of uptake of DNA across the membrane.

Organelles can be reintroduced into plant cells following electroporation by microinjection or polyethylene glycol-induced fusion. Such techniques are known in the art. Electroporation may also be used to affect reintroduction of organelles into plant cells. The DNA which plant cells take up can be stably maintained through subsequent generations. Such plant cells can undergo somatic embryogenesis and express genes from acquired DNA.

The invention contemplates the use of plant cells and organelles generally. Such cells and organelles may be derived from multicellular plants or from unicellular plants. The individual plant cells may carry their complete cell wall intact, or they may have a cell wall which has been partially or totally removed.

In one specific embodiment of the invention, the plant cells to be electrically treated are in the form of protoplasts. Protoplasts are cells from which the cell wall has been removed, leaving the plasma membrane as the cells' outermost boundary. This embodiment is applicable to any plant from which protoplasts can be made. These include both monocotyledonous and dicotyledonous plants and unicellular plants such as algae, yeast or other plants such as ferns.

Protoplasts are formed generally by incubating plant cells with enzymes such as cellulase and hemicellulase to to remove the cellulosic cell walls from the cells. This is done in an isosmotic medium so that the cells do not lyse after they have lost the mechanical support of the cell walls. Typically, the medium will contain high concentrations of sugar such as 500 to 600 mM sucrose, sorbitol, or mannitol. Other means of removing the cell walls of plant cells while retaining the integrity of the cells can be used and include chemical, mechanical or other enzymatic means.

DNA suitable for use in the present invention can be derived from any suitable source, including bacterial, fungal, plant or animal donor cells. The only requirement is that the donor cells contain the DNA which one desires to introduce into the plant cells. Such DNA

can be native to the donor cells or can be the product of recombinant DNA technology. Such DNA may be in the form of a plasmid, virus or organelle. Alternatively, only parts of plasmids, viruses, or organelles may be contained in the DNA. As a result of recombinant DNA technology, the DNA may carry exogenous DNA from a plant, fungal, animal or bacterial source. Exogenous DNA is defined for the purposes of the present invention as DNA which is originally derived from another organism.

The DNA can be prepared by any of a variety of techniques well-known in the art. For example, if the DNA is bacterial or is carried on a bacterial vector, it can be prepared by growing bacteria such as E. coli or A. tumafaciens. Bacteria are generally harvested from their liquid growth medium by means of centrifugation. Typically, they are then lysed by means of detergent, enzymes, or a combination thereof. Various techniques can be used to purify the class of DNA molecules desired. For example, if a plasmid is desired, a quick denaturation-renaturation can be used to irreversibly denature chromosomal DNA. The denatured DNA can then be precipitated with salt, leaving the native plasmid in solution. Optionally, the DNA can be purified using bouyant density centrifugation in cesium chloride with ethidium bromide. These and other techniques are well known in the art.

Suitable DNA for use in the present invention can be either linear or circular in physical form. Irrespective of the immediate source of the DNA, it can additionally contain exogenous DNA which has been acquired through recombinant DNA technology. So, for example, plasmid DNA can be used in the present invention which has been grown in yeast but which contains a bacterial drug-resistance gene, acquired via recombinant DNA technology. A suitable DNA molecule which may be used as a vector is derived from an Agrobacterium tumor inducing (Ti) plasmid. It is advantageous that the virulence genes of such a plasmid be inactivated or removed by mutation if

teratomous growth is not desired. It is also desirable that DNA molecules to be introduced contain the T-DNA region of Agrobacterium tumafaciens or A. rhizogenes. Such DNA region allows for the stable insertion of introduced DNA into the host plant's genome. Other means for accomplishing stable insertion of introduced DNA are compatible with the invention, including use of contiguous segments of DNA from the host to flank the DNA whose insertion is desired.

Generally, any ratio of DNA to cells or organelles may be used in the electrical treatment. However, it will often be desirable to maximize the efficiency of the process. Optimization of the ratio of cells to DNA can be performed for any particular set of circumstances by routine tests, as known to those skilled in the art. For example, it has been found that protoplasted carrot cells and DNA can be mixed at a ratio between about one microgram of DNA per $10^6$ cells to about 200 micrograms of DNA per $10^6$ cells. Preferably, they are mixed at a ratio of between about 5 and about 20 micrograms of DNA per $10^6$ cells.

A suitable medium for the electric treatment of the protoplasts or organelles is one which is buffered, contains magnesium, and is isosmotic. For example, Wong and Neumann's F-medium (Biochemistry and Biophysics Research Communications, Vol. 107, pp. 584-587, 1982; Krens et al. Nature 296, 72-74, 1981) has been found to be particularly well-suited for this purpose. Other media may of course be used and such determination can be made in any particular case by routine tests.

Generally, it is desirable to keep the mixture of plant cells or organelles and DNA cold before electrical treatment. Although the temperature range can be lowered, depending on the freezing temperature of the medium, suitable temperatures are below those at which degradative enzymes function and above those at which ice crystals form in the medium, for example, from about -15°C to about 10°C.

Generally, it is desirable to hold the mixture at a lowered temperature for a short period before electrical treatment; the period can range from a few minutes to a few hours and is not thought to be a critical parameter. Typically, the protoplast (or cell or organelle) and DNA mixture is kept at between about -10°C and 5°C for between about 5 and 10 minutes before and after electroporation. Although the applicants do not wish to be bound by any particular theory, it is believed that the short holding period allows for association of the membrane with the DNA, and increases the amount of DNA uptake.

The mixture is typically held at about 0°C up to room temperature during the electroporation, although the same ranges and considerations apply to the treatment as to the holding period. This may both reduce the chance of degradation of the DNA and keep the membrane in its more permeable conformation.

Electrical treatment can be carried out employing existing equipment. Generally, the electrical treatment's strength and duration are determined for a particular set of cell or organelle type and DNA source by routine testing. Optimization of these parameters are well within the skill of the art. For example, for carrot protoplasts and an Agrobacterium plasmid, it has been found that electrical pulses of a six microsecond duration with a two microsecond interval between each pulse are suitable. The field strength can vary from about 0.1 to about 5 kV/cm.

After electroporation, the plant cells can be incubated at temperatures and light regimes which are conducive to their growth. For example, carrot protoplasts are generally kept between about 20°C and about 30°C with a photoperiod of approximately 12 hours. The growth medium will generally contain plant hormones necessary for growth such as auxins and cytokinins, at concentrations of between about 0.1% and about 2%. At an appropriate time for the species being used, plant hormones can be removed from the growth medium to stimulate somatic embryogenesis. For example, at about 6 to 14 days after

protoplasts are made and electroporation performed, carrot plant cells become hormone-independent and plant hormones can be removed from the medium.

After electroporation, organelles can be reintroduced to protoplasts. This can be accomplished by any of the means known in the art, such as polyethylene glycol induced fusion and microinjection. Electroporation may also be used to make the plant cells more permeable to organelles.

Although the words "plant protoplast" have been used above, it is to be understood that full protoplasts are not necessary, but partially protoplasted cells or whole cells may also be used in this invention. It is also to be understood that the mechanism by which this invention works is not known. The use of the term "electroporation" for the process merely refers to the high-voltage electrical treatment of the cells described herein and does not presume a theory or mechanism.

The following examples are intended for illustrative purposes only and are not intended to limit the scope of this invention.

## EXAMPLES

### Example 1

This example sets forth the method employed for preparation of carrot protoplasts. It is essentially as described in Grambow et al. Planta, Vol. 103, pp. 348-355, 1972.

Rapidly growing, three-day old D. carota liquid suspension cultures were treated by the enzymes cellulase and hemicellulase to remove the cell wall. This was performed in a medium having sufficient osmotic pressure to maintain the cells or protoplasts intact without lysis, in this case due to 0.6 M mannitol. After enzymatic digestion, only the cell membrane remained as the outer boundary of the protoplast. Differential centrifugation in 0.5 M sucrose was used to separate the protoplasts from the degraded part of the cell wall. Protoplasts were finally resuspended in F medium: 20 mM $MgCl_2$, 140

mM NaCl, 5 mM KCl, 0.75 M $NaHPO_4$, 5 mM glucose, 0.5 M sucrose, pH 7.5.

Example 2

This example demonstrates the effect of various electrical field strengths on the uptake of radiolabeled DNA by carrot protoplasts.

Carrot protoplasts were prepared from suspension cultures and resuspended in F medium, as described in Example 1. Radiolabeled DNA was prepared by nick translation of plasmid pUCD9k3 to a specific activity of 1 x $10^8$ dpm/ug with [$^{32}$P]dTTP as described by Rigby et al., Journal of Molecular Biology, Vol. 113, pp. 348-355, 1977.

The protoplasts and radiolabeled DNA were mixed at a ratio of about 4 x $10^3$ DNA molecules per protoplast. In addition, calf thymus DNA (50 u/ml) was added as carrier. The mixture was incubated at 0°C for 5 minutes prior to being subjected to the electric field treatment. The precooled vessel in which the mixture was electrically treated was a "200 uM Precision™" having two platinum electrodes 20 microns in diameter separated by a distance of 200 microns. The power supply used was a "GCA/Precision Z-1000™". The field strength was varied over a range of 0 to 4 kV per centimeter. The total duration of the application of the electrical field was 36 microseconds, consisting of six 6us pulses.

After electrical treatment, the mixtures were kept at 0°C for 10 minutes, after which they were warmed to room temperature. DNAse I, obtained from Sigma Company, St. Louis, Missouri, was added to the mixture at a concentration of 0.4 ug/ul and digestion was performed for 30 minutes at 30°C to degrade all DNA remaining extracellularly. The protoplasts were then washed two times with Gamborg's B5 medium (Experimental Cell Research, Vol. 50, pp. 151-158, 1968) plus 0.5 M sorbitol, and then lysed with trichloroacetic acid by adding an equal volume of 10% solution. The lysates were filtered through glass fiber filters (Whatman GF/C) which retain precipitated macromolecules, and washed with additional trichloroacetic

acid. The radioactivity remaining on each filter, representing the DNA taken up by the protoplasts, was counted in a Beckman LS-7500 scintillation counter. Based on the specific activity of the labeled plasmid DNA, the number of plasmid molecules incorporated per protoplast was calculated. The results are shown in Table 1.

Table 1

| Field Strength (kv/cm) | Number of Pulses (6us) | Number of pUCD9k3 DNA molecules per protoplast |
|---|---|---|
| 0 | 0 | 1.6 |
| 0.5 | 6 | 9.5 |
| 1.0 | 6 | 3.6 |
| 1.5 | 6 | 2.2 |
| 2.0 | 6 | 2.3 |
| 4.0 | 6 | 5.4 |

The uptake of radiolabeled plasmid DNA was stimulated approximately seven fold by treating protoplasts for 36 microseconds at a field strength of 0.5 kV/cm.

Example 3

This example describes the method of regeneration of protoplasts after electroporation.

The protoplast and DNA mixture was removed from the electroporation vessel to the precooled lid of a plastic petri dish in 10 microliter aliquots and kept at 0°C for 10 minutes. An equivalent volume of Gamborg's B5 culture medium (20 g/l of sucrose, 36.4 g/l mannitol, 0.3 g/l ribose, 1 g/l casamino acids, 0.25 g/l $CaCl_2$, 1 mg/l 2,4-dichlorophenoxy acetic acid and 2 mg/l kinetin) was added to each aliquot. The lids were inverted to create hanging drop cultures, and incubated for 6-14 days at 26°C with a 12 hour photoperiod at 300 lux. The cells were then transferred to sterile polypropylene tubes and washed twice with Gamborg's B5 culture medium without hormones. The cells were resuspended in 10 ml B5 culture medium without hormones and transferred to 6 cm tissue culture plates. These cells were further incubated at 26°C with a 12 hour photoperiod.

Example 4

This example, like Example 2, demonstrates the effect of various electrical field strengths on the amount of DNA taken up by D. carota protoplasts. However, unlike Example 2, the means of detection of DNA which the protoplasts have incorporated is biological.

The DNA used in this example is pTiC58, an Agrobacterium tumor-inducing plasmid. When incorporated into a plant cell, pTiC58 DNA causes hormone-independent growth of somatic embryos, which have a teratoma-like appearance.

Protoplasts were prepared as in Example 1 and subjected to an electric field as in Example 2. However, plasmid pTiC58 DNA was added to each sample to a final concentration of 10 ug/ml, rather than adding radiolabeled DNA. The number of teratoma-like embryos was determined 45 days after electric pulse treatment.

The protoplasts were regenerated following their treatment with pTiC58 and electrical pulses, and then the number of protoplasts which were able to survive in the absence of hormones at 6 days post-electroporation to yield disorganized calli or teratoma-like growths was determined. (Cells which have not taken up pTiC58 DNA do not survive because they do not achieve hormone-independence until 7 days post-electroporation.) The frequency of hormone-independent regenerants indicates biologically the number of cells which were able to take up DNA and express it. The results of three different regimes are shown in Table 2.

When no DNA and/or no voltage were used, no teratoma-like somatic embryos were observed. Therefore, the observed changes in growth (hormone-independence and teratoma-like somatic embryos) are totally dependent upon both pTiC58 DNA and application of an electric field.

TABLE 2

| Field Strength | Days After Electric Field Applied that Hormones Removed From Medium | No. of Tetratoma-like Embryos per 10⁶ Cells per ug DNA |
|---|---|---|
| 1.3 | 14 | 130 |
| 2.0 | 6 | 121 |
| 3.8 | 14 | 163 |

Example 5

This example demonstrates that the electric field treatment of carrot protoplasts alone does not effect somatic embryogenesis of carrot.

Carrot protoplasts were prepared as in Example 1 and subjected to an electric field of 2 kV/cm for 36 microseconds in the apparatus described in Example 2. No DNA was added to the protoplasts in this experiment. The protoplasts were regenerated into somatic embryos as described in Example 3, removing plant hormones from the medium 14 days after electric treatment. Forty-five days after electroporation, the cells were compared to the non-electrically treated control cells. No substantial difference was observed between the two cultures, either in growth rate or in the number of embryos. At 130 days after electrical treatment, both treated and non-treated cultures had developed into normal plantlets. This shows that the electrical treatment causes no significant harm to the protoplasts or their regenerative capacity, nor does it cause teratoma-like growth.

Example 6

The following example demonstrates that the electrical treatment of protoplasts does not cause them to be hormone-independent.

Electrically pulsed and control non-pulsed protoplasts were regenerated as above, but the plant hormones were removed from the culture medium 6 days after electrical treatment. Forty-five days after electrical treatment, no significant growth was observed. This

shows that the hormone-independence of regenerants in Example 4 was due to the pTiC58 DNA and not the electrical treatment itself.

Example 7

This example demonstrates that the teratoma-like callus formed by carrot protoplasts which had been electrically treated to take up pTiC58 DNA make a pTiC58-determined metabolic product.

The plasmid pTiC58 encodes a gene for the enzyme nopaline synthase. This enzyme converts arginine to nopaline. To determine whether the teratoma-like callus contained nopaline, two assays were used: paper electrophoresis and a bioassay.

Identical weights of carrot plantlet and teratoma tissue were homogenized by squeezing between two metal plates. Cell debris was removed by centrifugation. 100 microliters of each of these tissue extracts as well as standards of nopaline and arginine were applied directly to Whatman 3MM™ paper saturated with electrophoresis buffer (Otten, L. and Schilperoort, R. (1978) Biochem. Biophys. Acta, 527, 497-500). Electrophoresis was carried our for 60 to 70 minutes at 300 volts per 15 centimeters. The paper was dried and then sprayed with a fresh alcoholic phenanthrenquinone solution.

When viewed under short wave UV light, the compounds containing free amine groups flouresced. A flourescent spot having identical mobility with nopaline was found in homogenates from teratoma-like callus; however, no such spot was detectable in the normal carrot tissue homogenate. This confirms the presence of pTiC58 DNA in the teratoma-like callus.

To detect nopaline via bioassay, 50 mg each of tissue from electrically treated teratoma-like callus and normal carrot plantlet were embedded in M9-agar medium in a petri dish. The medium is a buffered salts solution and contains no reduced nitrogen or carbon source; therefore, it cannot support bacterial growth. Agrobacterium tumefaciens LBA 4301 is a strain which can metabolize nopaline, and can use it as both a nitrogen and carbon source. A suspension of this

strain was spread on the surface of the agar petri plates containing the plant tissue.

After a 48 hour incubation at 30°C, plates were examined for bacterial growth. The plate containing teratomous tissue supported abundant bacterial growth, whereas the normal carrot plantlet tissue supported little or no growth. This suggests that the teratomous tissue can make nopaline. This further confirms the presence of pTiC58 DNA in the teratomous tissue.

Example 8

This example demonstrates that the teratoma-like callus formed by carrot protoplasts which had been electrically treated to take up pTiC58 made a pTiC58-determined gene product.

The plasmid pTiC58 encodes a gene for the enzyme nopaline synthase. The presence of the enzyme activity in the teratoma-like callus was detected by adding the substrates arginine, 2-ketoglutarate, and NADH (the reduced form of nicotinamide adenine dinucleotide) to a crude extract of plant tissue.

The crude extract was prepared by grinding 10 mg of tissue in an equal volume of a buffer containing 10 mM Tris, 1 mM EDTA, 0.1% cysteine-HCl, pH 8.0. Cell debris was removed by centrifugation.

After the substrates were added to the plant extracts, they were incubated at 37°C for 4 hours. Twenty ul of the reaction mixture was then applied directly to buffer-saturated Whatman 3MM™ paper. The electrophoresis was performed and the compounds detected as in Example 7. Extracts made from normal carrot plantlet produced no nopaline whereas teratomous tissue produced substantial amounts of nopaline. This is consistent with the notion that the teratoma-like callus contains pTiC58 DNA and expresses nopaline synthase.

Example 9

This example demonstrates the physical presence of DNA sequences derived from pTiC58 in teratoma-like callus formed upon electrically treating mixtures of carrot protoplasts and pTiC58 DNA.

Total DNA was isolated from normal carrot plantlets and from teratoma-like callus derived from pTiC58 transformed carrot protoplasts, as follows. The plant tissue was ground in liquid nitrogen and the powdered tissue was added to an equal volume of buffer consisting of 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 50 mM NaCl, 2% SDS, and 2 ml/l beta-mercaptoethanol. As soon as the mixture had thawed, it was extracted with an equal volume of phenol:chloroform, 1:1 (v/v). Separation of the aqueous and organic phases was facilitated by centrifugation (10,000 rpm for 10 min.). The aqueous layer was removed and reextracted with phenol:chloroform. The resulting aqueous layer was transferred to a beaker and overlayered with three volumes of 95% ethanol. High molecular weight DNA was spooled from the interface of the aqueous and the organic layers. The spooled DNA was washed with 70% ethanol, dried in vacuo, and resuspended in 10 mM Tris-HCl, pH 7.0, 1 mM EDTA.

DNA samples were cleaved with 4 units restriction enzyme BamHI for 4 hours, and the DNA fragments were subjected to agarose gel electrophoresis. The DNA fragments were transferred from the agarose gel to nitrocellulose filter paper as described by Southern (Journal of Molecular Biology, Vol. 98, pp. 503-517, 1975).

The T-DNA containing restriction enzyme KpnI or III fragment (23 kilobasepairs) was isolated and radiolabeled to 1 x $10^8$ dpm/ug using alpha [$^{32}$P]-nucleotide and the enzyme DNA polymerase I.

To hybridize, the radiolabeled T-DNA was denatured and mixed with the nitrocellulose which contained the DNA from the agarose gel in 3 x SSC buffer (Denhardt, Biochemistry and Biophysics Research Communications, Vol. 23, pp. 641-646, 1966) containing 46% formamide. This hybridization was carried out at 37°C for 48 hours. The filter was then washed with buffer, dried, and autoradiographed for up to three weeks on Kodak XAR film at -70°C.

The radiolabeled T-DNA probe hybridized to DNA fragments from the teratoma-like callus tissue, but not to any fragments from the

normal carrot plantlet. This hybridization confirms that the electrically and pTiC58-treated protoplasts took up pTiC58 DNA and were able to maintain the T-DNA stably through subsequent cell divisions.

While certain specific embodiments of the invention have been described with particularity herein, it will be recognized that various modifications thereof will occur to those skilled in the art. Therefore, the scope of the invention is intended to be limited solely by the scope of the appended claims.

CLAIMS

1. A method of introducing DNA into plant cells or organelles comprising:

mixing the plant cells or organelles and the DNA in a suitable medium, and

treating the plant cells or organelles and DNA mixture by subjecting it to a pulsed electric field of at least about 0.01 kV/cm.

2. The method of claim 1 wherein the plant cells into which DNA is introduced are protoplasts.

3. The method of claim 1 wherein the mixture of plant cells or organelles and DNA are preincubated at between about -15°C to about 10°C for between about 2 to about 60 minutes.

4. The method of claim 1 wherein the mixture of plant cells or organelles and DNA are kept at between about -10°C and about 5°C while being subjected to the electric field.

5. The method of claim 1 wherein the plant cells or organelles are derived from a dicotyledonous plant.

6. The method of claim 1 wherein the plant cells or organelles are derived from a monocotyledonous plant.

7. The method of claim 1 wherein the electrically treated mixture of plant organelles and DNA is reintroduced into plant cells.

8. The method of claim 1 wherein the electrically treated mixture of plant cells and DNA is incubated in the presence of plant hormones to provide a transformed plant cell.

9. The method of claim 8 wherein the transformed plant cells are stimulated to undergo somatic embryogenesis by removing plant hormones from the incubation medium at an appropriate time.

10. The method of claim 1 wherein the DNA introduced into the plant cells or organelles is circular.

11. The method of claim 10 wherein the circular DNA is a plasmid from the genus Agrobacterium.

12. The method of claim 10 wherein the DNA contains T-DNA sequences derived from Agrobacterium.

13. The method of claim 1 wherein the DNA introduced is a linear piece of DNA.

14. The method of claim 13 wherein the DNA contains T-DNA sequences derived from Agrobacterium.

15. The method of claim 11 wherein the virulence genes of the Agrobacterium plasmid have been inactivated.

16. The method of claim 12 wherein the virulence genes of the Agrobacterium derived DNA have been inactivated.

17. The method of claim 14 wherein the virulence genes of the Agrobacterium derived DNA have been inactivated.

18. The method of claim 10 wherein the DNA carries an exogenous piece of DNA from a plant source.

19. The method of claim 10 wherein the DNA carries an exogenous piece of DNA from a bacterial source.

20. The method of claim 10 wherein the DNA carries an exogenous piece of DNA from an animal source.

21. The method of claim 10 wherein the DNA carries an exogenous piece of DNA from a fungal source.

22. The method of claim 13 wherein the DNA carries an exogenous piece of DNA from a plant source.

23. The method of claim 13 wherein the DNA carriers an exogenous piece of DNA from a bacterial source.

24. The method of claim 13 wherein the DNA carriers an exogenous piece of DNA from an animal source.

25. The method of claim 13 wherein the DNA carries an exogenous piece of DNA from a fungal source.

26. The method of claim 1 wherein the DNA introduced is a viral DNA.

27. The method of claim 1 wherein the DNA introduced is an organelle DNA.

28. A method of introducing DNA into plant cells or organelles comprising:

mixing the plant cells or organelles and the DNA together in a suitable medium, and

treating the plant cells or organelles and DNA mixture by subjecting it to a pulsed electrical field between about 0.01 kV/cm and about 5 kV/cm for between about 20 microseconds and about 400 microseconds.

29. The method of claim 28 wherein the electrically treated mixture of plant cells and DNA is incubated at between about 20°C and about 30°C for at least about 5 days in the presence of plant hormones.

30. The method of claim 28 wherein the electrically treated mixture of plant organelles and DNA is reintroduced into plant cells.

31. The method of claim 30 wherein the organelles are reintroduced into plant cells by a means selected from the group of microinjection, electroporation, and polyethylene glycol-induced fusion.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/00974

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4): C12N 15/00 C12N 5/00 C12N 1/00

U.S. CL: 435/172.3 435/240 435/317

**II. FIELDS SEARCHED**

Minimum Documentation Searched [4]

| Classification System | Classification Symbols |
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Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Computer Search CAS, Biosis 1969 to Present: Electroporation

**III. DOCUMENTS CONSIDERED TO BE RELEVANT** [14]

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No. [18] |
|---|---|---|
| X<br>Y | Plant Cell Reports, Volume 4, issued December 1985, (New York, New York, USA), Langridge et al., "Electric field mediated stable transformation of carrot protoplasts with naked DNA", pages 355-359, see pages 356 and 358 in particular. | 1-5,8-12, 19,28,29<br>6,7,13-18, 20-27,30, 31 |
| X | Trends in Biotechnology, Volume 3, issued August 1985, (Amsterdam, The Netherlands), N. Yang, "Transient gene expression in electroporated plant cells", pages 191-192, see page 191 in particular. | 1,2,5,6, 10,19,26 |
| X<br>Y | Proceedings of the National Academy of Sciences USA, Volume 82, issued September 1985, (Washington, DC, USA), Fromm et al., "Expression of genes transferred into monocot and dicot plant cells by electroporation", pages 5824-5828, see pages 5824 and 5825 in particular. | 1-6,8,10, 12,19,26, 28,29<br>7,9,11,13-18,20-25, 27,30,31 |

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search [2] | Date of Mailing of this International Search Report [2] |
|---|---|
| 26 June 1987 | 08 JUL 1987 |
| **International Searching Authority [1]** | **Signature of Authorized Officer [20]** |
| ISA/US | David T. Fox |

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No [18] |
|---|---|---|
| X<br>Y | Nature, Volume 319, issued February 1986, (London, England), Fromm et al., "Stable transformation of maize after gene transfer by electroporation," pages 791-793, see page 792 in particular. | 1-4,6,8, 10,12,19, 26,28,29<br>5,7,9,11, 13-18,20-25,27,30, 31 |
| X<br>Y | Plant Cell Reports, Volume 5, issued January 1986, (New York, New York, USA), Nishiguchi et al., "Electroporation-mediated infection of tobacco leaf protoplasts with tobacco mosaic virus RNA and cucumber mosaic virus RNA," pages 57-60, see page 58 in particular. | 1-5,8,26, 28,29<br>6,7,9-25, 27,30,31 |
| Y | Bioelectrochemistry and Bioenergetics, Volume 12, issued April 1984, (Lausanne, The Netherlands), Berg et al., "Possibilities of cell fusion and transformation by electrostimulation," pages 119-133, see pages 119 and 126 in particular. | 1-31 |
| Y | Biochemical and Biophysical Research Communications, Volume 107, issued July 1982 (New York, New York, USA), Wong et al., "Electric field mediated gene transfer," pages 584-587, see pages 585-587 in particular. | 1-31 |
| Y | The EMBO Journal, Volume 1, issued July 1982, (Oxford, England), Neumann et al., "Gene transfer into mouse lyoma cells by electroporation in high electric fields," pages 841-845, see page 842 in particular. | 1-31 |
| Y | Proceedings of The National Academy of Science USA, Volume 81, issued November 1984, (Washington, DC, USA), Potter et al., "Enhancer-dependent expression of human K immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation", pages 7161-7165, see pages 7161 and 7162 in particular. | 1-31 |
| Y | Nature, Volume 296, issued March 1982, (London, England), Krens et al., "In vitro transformation of plant protoplasts with Ti-plasmid DNA", pages 72-74, see page 73 in particular. | 1-31 |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No [18] |
|---|---|---|
| Y | The EMBO Journal, Volume 3, issued December 1984, (Oxford, England), Paszkowski et al., "Direct gene transfer to plants", pages 2717-2723, see page 2721 in particular. | 1-31 |
| Y | The EMBO Journal, Volume 2, issued December 1983, (Oxford, England), Zambryski et al., "Ti-plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity", pages 2143-2150, see pages 2144 and 2145 in particular. | 15-17 |
| Y | Science, Volume 190, issued August 1975, (Washington, DC, USA), Vasil et al., "Induced transfer of higher plant chloroplasts into fungal protoplasts," page 680. | 7,30,31 |